

# MAB L9E10 to Blood Group H2 Antigen Binds to Colon Cancer Stem Cells and Inhibits Tumor Cell Migration and Invasion

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The functions of the precursor H antigen for ABO blood group antigens are still not fully understood, particularly in cancer cells. In this study, we used hybridoma technology and NSY human colon cancer cells as an immunogen to generate a monoclonal antibody designated as MAb L9E10. The binding antigen of MAb L9E10 was identified as blood group (BG) H2 antigen using carbohydrate array and erythrocyte agglutination assays. In immunofluorescence study, we found that BG-H2 was expressed on the surfaces of both colon cancer stem cells and their differentiated progeny. In a functional study, we observed that MAb L9E10 inhibited tumor cell migration and invasion at a concentration of 10  $\mu\text{g}/\text{mL}$  *in vitro*. This result suggests that MAb L9E10 could be used to study cancer biology, particularly cancer stem cell biology. In addition, it is potentially useful for studying gastric diseases caused by *Helicobacter pylori* bacteria, with attachment to human gastric epithelial cells mediated by blood group antigens Lewis b and H2. Finally, MAb L9E10 is an ideal biological reagent for identifying Bombay blood type in which erythrocytes have no BG-H2 antigen expression.

## Introduction

**B**LOOD GROUP (BG) H ANTIGEN contains four subtypes, H1–H4, based on the structure of carbohydrate chains.<sup>(1)</sup> Generally, BG-H2 and BG-H4 antigens are expressed on red blood cells and vascular endothelium, and BG-H1 and BG-H3 antigens are found in epithelial cells of intestine and lungs and in secretory cells of the submaxillary gland.<sup>(2)</sup> In normal colon, BG-H1 antigen, but not BG-H2 or BG-H3/4 antigens, was detected in epithelial cells.<sup>(3–6)</sup> In contrast, BG-H2 antigen was aberrantly expressed in cancer tissues of the proximal and distal colon from secretors.<sup>(3)</sup> Negative expression of BG-H1 was also frequently observed in tumor cells and correlated with tumor progression.<sup>(1,7)</sup> Tumor metastases are the major cause of death in cancer patients. Studies have found that BG antigens including H antigen can be present on epidermal growth factor receptor (EGFR), integrins, cadherins, and CD-44 (a cell-surface glycoprotein), which are involved in cell proliferation, cell-cell interaction, cell adhesion, and motility as well as angiogenicity.<sup>(1,8–10)</sup> These results imply that BG antigens including H antigen play a role in tumor cell migration and invasion. In addition, BG-H2 antigen was detected on early stage CD34+ hematopoietic progenitors and normal stem-like cells of the breast gland.<sup>(2,11)</sup> BG-H2 is believed to be a marker of normal stem cells. However, the ex-

pression of BG-H2 in cancer stem cells (CSCs) has not been studied. The exact functions of BG-H2 in tumor cells need to be further investigated.

In this study, we generated a monoclonal antibody designated as L9E10 using human colon cancer NSY cells as an immunogen. We also identified the binding antigen of MAb L9E10 using a carbohydrate array and examined the expression of BG-H2 antigen in colon CSCs, which are hypothesized to be tumor-initiating cells.<sup>(12,13)</sup> Finally, we explored the effects of MAb L9E10 on NSY tumor cell migration and invasion *in vitro*.

## Materials and Methods

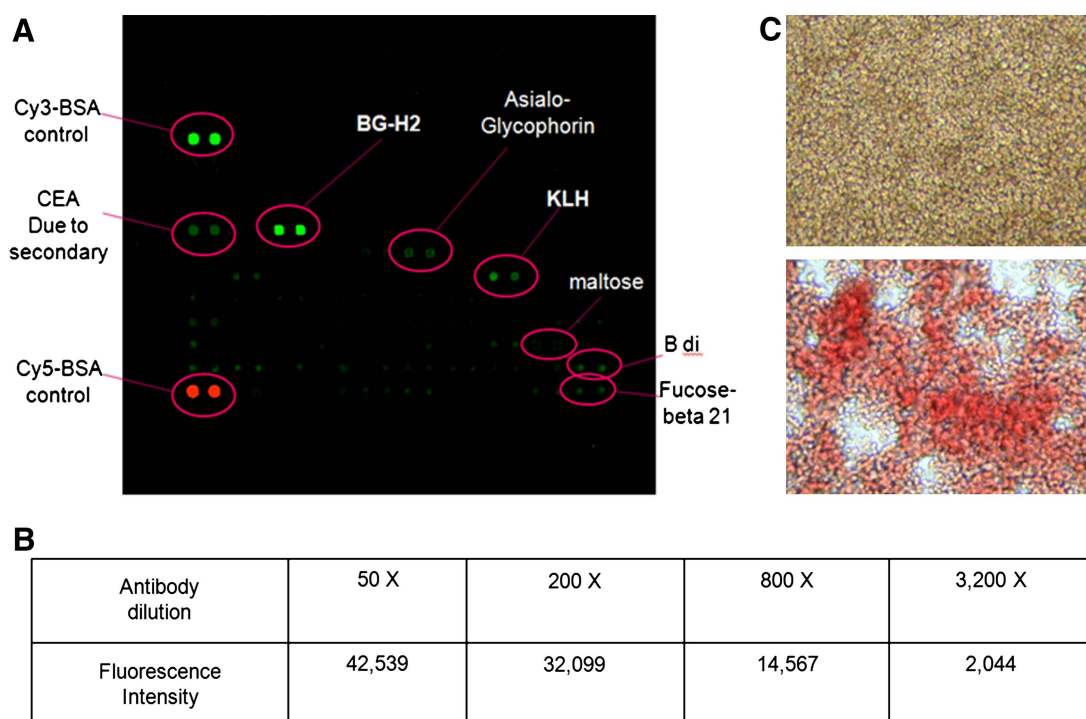
### Generation of monoclonal antibodies

To identify tumor cell surface specific biological markers using hybridoma technology, we injected Titermax Gold adjuvant (Sigma Chemical, St. Louis, MO) and NSY cells ( $1 \times 10^6$ ) into 6-week-old female BALB/c mice peritoneally once a week for 4 weeks. Three days before euthanasia, the mouse was boosted with the same doses of the adjuvant and tumor cells used above. Spleen cells from a mouse with serum titer >4000 were used for fusion. A hybridoma library was established by fusion of spleen cells from the immunized BALB/c mice with myeloma cells (P3/x63.Ag8) at a ratio of 5:1 in

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**FIG. 1.** Identification of MAb L9E10 binding antigen. (A) Carbohydrate array slide stained with MAb L9E10 mouse ascites at 800 $\times$  dilution. (B) Quantitative data of carbohydrate array slide stained with MAb L9E10 at different concentrations. (C) Agglutination assay of blood type O erythrocytes with MAb L9E10. Upper panel, control by adding equal volume (50  $\mu$ L) of PBS to 5% blood type O erythrocytes; lower panel, agglutinated red blood cells after adding equal volume (50  $\mu$ L) of 4000 $\times$  diluted MAb L9E10 mouse ascites and incubated at room temperature for 20 min.

polyethylene glycol (PEG)-1500 (Sigma Chemical) following standard procedures.<sup>(14)</sup> Briefly, freshly harvested spleen cells and fusion partner P3/x63.Ag8 cells were co-pelleted by centrifugation and fused by adding PEG-1500 solution to the pellet. Fused cells were centrifuged, resuspended in Iscove's modified Dulbecco's medium containing 20% (v/v) FCS and  $1 \times 10^{-5}$  mol/L hypoxanthine-aminopterin-thymidine (Sigma Chemical), and aliquoted into 96-well plates. The hybridomas were allowed to grow for 10 to 15 days, and the supernatants were then collected for screening. Screening was done in the NSY cells with an immunofluorescence staining assay. The supernatants containing an antibody that bound to the surface of NSY cells were further screened in a carbohydrate array for identification of their binding antigens. MAb L9E10 was purified using protein G Sepharose according to the manufacturer's instruction (Amersham Biosciences, Piscataway, NJ). Briefly, the supernatant from the hybridoma culture was centrifuged at 14,000  $g$  for 20 min at 4 $^{\circ}$ C and filtered through a 0.22  $\mu$ m filter to remove fine particles; the pH was adjusted to 7.0 using equilibration buffer (1 mol/L Tris, pH 9.0). The supernatant was passed through a protein G column, and the column was then washed with binding buffer (50 mmol/L  $\text{Na}_2\text{PO}_4$ , 500 mmol/L NaCl, pH 6.0), before eluting the antibody with glycine (0.1 mol/L, pH 2.7). The antibody was collected and neutralized in a neutralization buffer (1 M Tris/HCl, pH 9.0).

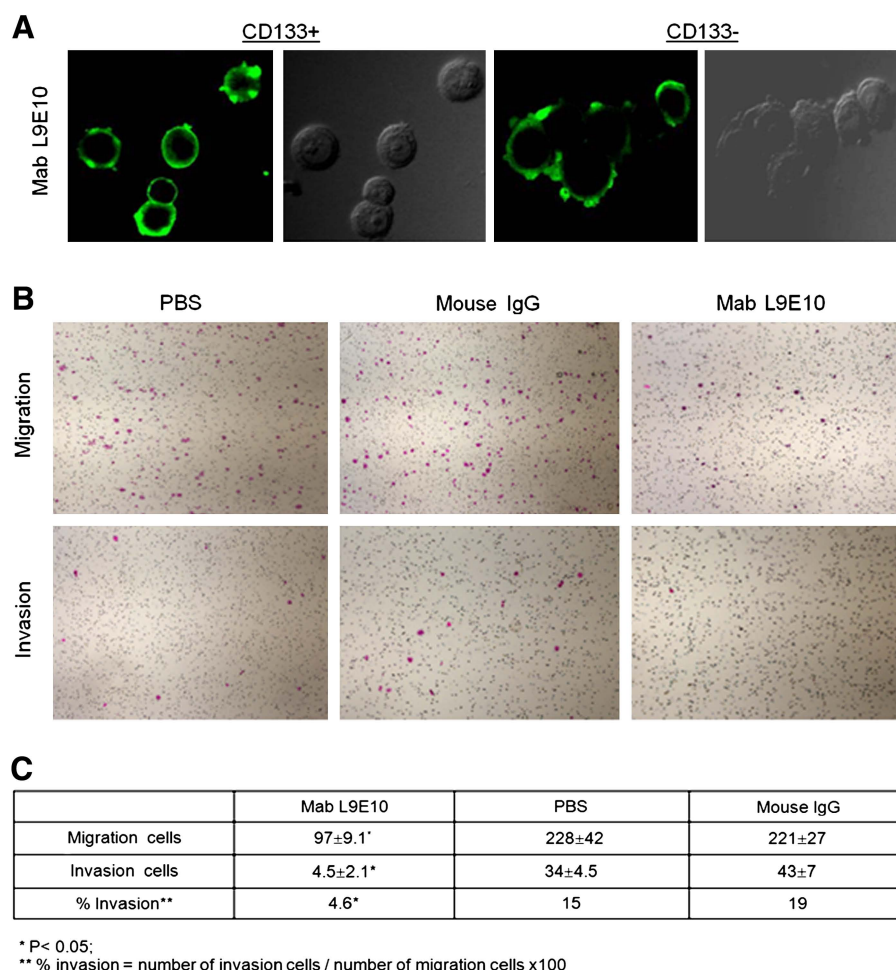
#### Monoclonal antibody ID typing

Isotype of MAb L9E10 was determined using a clonotyping kit (cat. no. 5300-05, Southern Biotech, Birmingham, AL) fol-

lowing the manufacturer's instructions. In brief, 0.1 mL diluted capture antibody (5–10  $\mu$ g/mL) was added to each well of the ELISA plate. The plate was incubated at 4 $^{\circ}$ C overnight. After emptying the wells and washing three times with PBS containing 0.05% Tween-20, blocking buffer (PBS containing 1% bovine serum albumin) was dispensed into each well. The MAb L9E10 (0.1 mL; 10  $\mu$ g/mL) was added to each well after washing and the plate was incubated for 1 h at room temperature with gentle shaking. After washing, 0.1 mL of dilute HRP-labeled detection antibody in BSA was added to the appropriate wells of the plate and incubated for 1 h at room temperature with gentle shaking. The substrate solution (0.1 mL) was added to each well of the plate after three washes with PBS. Finally, the plate was read with a Synergy HT Multi-Detection microplate reader (Bio-Tek, Winooski, VT) at 405 nm at the time points of 10 and 20 min after substrate addition.

#### Carbohydrate array profiling

To identify MAb L9E10 binding antigen, we evaluated binding on a carbohydrate array containing 163 different glycoproteins and neoglycoproteins (for a complete list of array components, see Supplementary Table 1). Fabrication of arrays and assessment of antibody binding was carried out as reported previously with minor modifications.<sup>(15,16)</sup> Briefly, the array was blocked with 3% bovine serum albumin (BSA)/PBS for 2 h, incubated with antibodies at four different dilutions for 2 h at room temperature, washed with PBS, and then incubated with 50  $\mu$ L of Cy3-labeled anti-mouse immunoglobulin IgG (Jackson ImmunoResearch



**FIG. 2.** Expression of BG-H2 antigen in colon CSCs and biological effect of MAb L9E10 on tumor cell migration and invasion. (A) Immunofluorescence staining of human colon cancer NSY cancer stem (CD133+) cells and differentiated (CD133-) cells with 2000× diluted MAb L9E10 mouse ascites. (B) Images of human colon cancer NSY cell migration and invasion assay. Left panel control, 10  $\mu$ L PBS added to equivalent amount of antibodies; middle panel control, 10  $\mu$ g/mL normal mouse IgG; right panel, MAb L9E10 (10  $\mu$ g/mL). The larger pink dots are migration and invasion tumor cells. Tiny black dots are 8 micron membrane pores. (C) Quantitative analysis of MAb L9E10 on colon cancer NSY cell migration and invasion.

Laboratories, West Grove, PA) in 3% BSA for 1.5 h. After washing and drying, the slides were scanned on a GenePix scanner (GenePix 4000A Microarray Scanner, Molecular Devices, Union City, CA). The fluorescence was quantified by using Gene-Pix Pro 6.0 software with a GenePix Array List file. The value for each array component was obtained by averaging the background corrected median intensities of the two replicate spots. Full array data can be found in Supplementary Table 2.

#### Red blood cell agglutination assay

To further confirm that MAb L9E10 reacts to H antigen, we performed a red blood cell agglutination assay. Anti-coagulated blood type O red cells were diluted 20× with PBS. The diluted type O blood cells (50  $\mu$ L) and MAb L9E10 (4000× diluted ascites) were added to a well of a 96-well plate. The mixture of red blood cells and MAb L9E10 was incubated at 37°C for 10 min and observed under the microscope.

#### Isolation of colon cancer stem cells and immunofluorescence staining of MAb L9E10

CD133 Indirect Cell Isolation Kit (Miltenyi Biotec, Auburn, CA) was used to fractionate colon CSCs from human colon carcinoma NSY cells following the manufacturer's standard procedures. For this procedure, after trypsinization,  $1 \times 10^7$  cells were resuspended in 100  $\mu$ L of labeling (separation) buffer (PBS without  $\text{Ca}_2^+$  and  $\text{Mg}_2^+$ , 0.5% bovine serum albumin, 2 mM EDTA, pH 7.2). Biotinylated antibody against CD133/1 (10  $\mu$ L) was added and refrigerated for 10 min. After washing, the cell pellets were suspended in 80  $\mu$ L of labeling buffer and 20  $\mu$ L of anti-biotin MicroBeads and incubated at 4°C for 15 min; the cells ( $1 \times 10^7$ ) were suspended in 500  $\mu$ L of the separation buffer and loaded onto the column for magnetic separation. The isolated human colon cancer NSY CSCs (CD133+) and differentiated (CD133-) cells were cultured at 37°C overnight, fixed with 10% formalin, and stained with MAb L9E10 at 2000× dilution mouse ascites. The stained

slides were visualized with the Olympus FV 1000 microscope (Tokyo, Japan).

### Tumor cell migration and invasion assays

To test the biological effect of MAb L9E10 on tumor cell migration and invasion, we performed a cell migration and invasion assay using the BioCoat Matrigel invasion chamber (PN: 354481, Becton, Dickinson, Franklin Lakes, NJ) in accordance with manufacturer's instruction. Briefly,  $1 \times 10^5$  NSY cells in 0.5 mL medium with 10  $\mu\text{g}/\text{mL}$  MAb L9E10 or 10  $\mu\text{g}/\text{mL}$  normal mouse IgG were added to each chamber (duplicate for each treatment) after rehydration and cultured in a humidified tissue culture incubator at 37°C with 5%  $\text{CO}_2$  for 72 h. The cells were counted under a microscope after removing noninvading cells by scrubbing the top surface of the membrane with a dry cotton swab, fixing with 4% neutral formalin, and staining with hematoxylin and eosin.

### Results and Discussion

Malignant transformation of normal cells is often associated with alteration of cell surface carbohydrate antigens.<sup>(17,18)</sup> Altered cell surface carbohydrate antigen expression, such as that of blood group antigens, has been correlated with tumor development and progression.<sup>(19–21)</sup> Blood group antigens are a major component of epithelial and endothelial cell surface antigens. In this study, we have generated a monoclonal antibody designated as L9E10 by immunizing BALB/c mice with human NSY colon cancer cells. MAb L9E10 binding antigen was identified as BG-H2 (Fig. 1A). Fluorescent signals on the carbohydrate slide were detected down to a 1:3200 dilution of MAb L9E10 mouse ascites (Fig. 1B). A few other weak signals at a higher concentration (800 $\times$ ) of MAb L9E10 are most likely due to contamination of endogenous antibodies in the ascites. To further confirm that the binding antigen was a BG-H2 antigen, we performed an erythrocyte agglutination assay with blood type O erythrocytes in which BG-H2 was dominantly expressed. MAb L9E10 efficiently agglutinated the red blood cells at a concentration of 4000 $\times$  dilution of mouse ascites (Fig. 1C). The results shown in Figure 1 clearly demonstrate that MAb L9E10 specifically reacts to BG-H2 antigen. Using a clonotyping kit, idiotype of MAb L9E10 was verified as an IgG2a with kappa light chain.

BG-H2 is considered an oncofetal antigen since it is frequently expressed in fetal and cancer tissues.<sup>(22,23)</sup> It is also reported that BG-H2 is expressed in early stage hematopoietic cells and embryonic stem cells.<sup>(24)</sup> However, the expression of BG-H2 in CSCs, particularly in colon cancer CSCs, is unknown. In this study we found that BG-H2 antigen was overexpressed on the surface of colon CSCs (CD133+) (Fig. 2A, left panels) as well as colon cancer differentiated (CD133-) cells (Fig. 2A, right panels). The intensive MAb L9E10 staining on the surfaces of both colon CSCs and differentiated cells led us to explore the biological effect of BG-H2 on tumor cell migration and invasion. As demonstrated in Figure 2B, MAb L9E10 effectively inhibits human colon cancer NSY cell migration and invasion. Quantitative data of MAb L9E10 on NSY cell migration and invasion show that MAb L9E10 at 10  $\mu\text{g}/\text{mL}$  significantly suppresses tumor cell migration and invasion. Specifically, there were  $97 \pm 9.1$  migration cells and  $4.5 \pm 2.1$  invasion cells with the MAb compared with  $228 \pm 42$  migration and  $34 \pm 4.5$  invasion cells in PBS control as well as

$221 \pm 27$  migration and  $43 \pm 7$  invasion cells in normal mouse IgG control at 72 h. Furthermore, the percent invasion in MAb L9E10 is much lower (4.6%) compared to those of PBS and normal mouse IgG (15% and 19%, respectively). Notably, all the differences in the numbers of migration and invasion cells and the invasion percentage are significantly different between MAb L9E10 and controls with PBS and normal mouse IgG (Fig. 2C). Our results clearly indicate that the MAb L9E10 binding antigen BG-H2 plays a role in tumor cell metastasis and concur with the observations that blood group antigens can be presented by integrins, cadherins, CD44, and EGFR that have a strong influence on cell motility.<sup>(25)</sup>

In summary, we have generated an anti-BG-H2 antibody L9E10, discovered the expression of BG-H2 antigen in colon CSCs, and demonstrated the inhibition effect of MAb L9E10 on colon cancer NSY cell migration and invasion. Despite the fact that antibodies to BG-H2 have been reported,<sup>(26–30)</sup> some of the antigen was identified based only on agglutination assays of red blood cells and Ulex Europaeus lectin assays.<sup>(28–30)</sup> In this study, we used a carbohydrate array to identify the MAb L9E10 binding antigen as a BG-H2. Thus, the new MAb L9E10 can be added to the short list of anti-BG-H2 monoclonal antibodies. As a biological reagent, MAb L9E10 may be useful in the study of cancer biology, particularly cancer stem cell biology as well as gastric diseases caused by *H. pylori* bacteria, with attachment to human gastric epithelial cells mediated by blood group antigens Lewis b and H2.<sup>(31,32)</sup> Additionally, it can be used for the identification of the Bombay blood type in which erythrocytes have no BG-H2 antigen expression.<sup>(28)</sup>

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